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**14. ABSTRACT**

Animal research suggests that reactivation (retrieval) of a consolidated memory can return it to a labile state from which it must be restabilized in order to persist. This stabilization process has been termed "reconsolidation", and various behavioral and pharmacological interventions have been found to modify or block it. The aim of this project is to create an experimental assay in the form of an optimal Pavlovian differential fear-conditioning paradigm, within which the relative strengths of various pharmacological and behavioral, reconsolidation-blocking interventions can be tested. Thus far, we have completed data collection for two groups: pharmacological, i.e., propranolol, and behavioral, i.e., delayed extinction. Data from the propranolol group demonstrate that participants show differential conditioning learning on Day 1, supporting the validity of our modified fear-conditioning paradigm. However, propranolol administration at the time of memory reactivation failed to decrease the fear memory, as indexed by skin conductance. These findings are communicated in a manuscript (attached), which will be submitted for publication to Psychophysiology later this month. Data from the behavioral group are presently being analyzed and prepared for publication. We are presently recruiting participants to receive a new candidate pharmacological intervention, mifepristone (RU-486), within the same fear-conditioning paradigm.

**15. SUBJECT TERMS**- Post-traumatic stress disorder; reconsolidation; fear conditioning; psychophysiology

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## 1. INTRODUCTION

Background: Animal research suggests that reactivation (retrieval) of a consolidated memory can return it to a labile state from which it must be restabilized in order to persist. This stabilization process has been termed "reconsolidation," and various pharmacological and non-pharmacological interventions can block it. This ability offers novel therapeutic possibilities for PTSD. To date, few human studies have been conducted on the mechanism of memory reconsolidation blockade; some of them have yielded positive results but their clinical relevance to the problem of PTSD remains very limited. Two recent studies performed in healthy human subjects have addressed the question of memory reconsolidation blockade using fear-conditioning paradigms, which are highly relevant to PTSD. Both studies demonstrated that fear memory could be eliminated via the mechanism of reconsolidation. The first study (Kindt et al. 2009) demonstrated the phenomenon using a beta-blocker, propranolol, whereas the other study (Schiller et al. 2010) obtained similar results using a non-pharmacological/behavioral intervention that combines an extinction protocol within a reconsolidation paradigm (delayed extinction). Given that both studies quickly and completely abolished fear responses, this floor effect prevents us from using their study design in order to test the relative strengths of various reconsolidation-blocking (or memory updating) interventions. In other words, if propranolol or delayed extinction totally eliminates the conditioned fear response, no other intervention could be found to be superior.

Goals: The specific aim of the present project is to create an experimental assay in the form of an optimal Pavlovian differential fear conditioning paradigm within which the relative strengths of various novel behavioral and pharmacological reconsolidation-based interventions can be compared. In order to accomplish this, we designed a new experimental protocol that is free of floor effects. Specifically, we are testing the following modifications to existing experimental designs: 1) use of a more highly "prepared" (i.e., danger-signaling) conditioned stimulus (CS); 2) recruitment of more sensitive subjects; 3) selection of only subjects who acquire strong conditioned responses (CRs) during conditioning for further participation, and 4) use of additional probes for the presence of the latent CR, viz., renewal and savings in addition to spontaneous recovery and reinstatement.

General procedure: The protocol consists of four distinct visits taking place over the course of a month. On Day 1 (habituation and acquisition), subjects view video clips of three rooms (contexts) different in color and content, presented on a 42" high definition television. The stimuli are three different videos of tarantulas, one presented in the context of each room. Two of the three tarantulas serve as the two CS+s and the third as the CS-. Each CS+ presentation is sometimes followed by shock (i.e. reinforced); the CS- is never be followed by shock. During this first session, each CS is presented twice in an unreinforced manner (habituation phase). Next, during the acquisition phase, there are 8 presentations of each CS, with 5 of each CS+ presentations followed by shock (i.e. 62.5% reinforcement). The acquisition phase takes place in Context A. On Day 2 (intervention), subjects are assigned an intervention, whether pharmacological (i.e., 40mg propranolol, 1800mg mifepristone) or behavioral (i.e., delayed extinction). Drug administration is followed 90 minutes later by a single, unreinforced presentation of one of the two CS+, designated the reactivated CS+ (CS+R). For the behavioral intervention, participants are exposed to a single, unreinforced presentation of the CS+R without receiving a pill, followed 10 minutes later by 10 further unreinforced CS+R presentations, 11

unreinforced presentations of the remaining CS+ (designated the CS+ with no intervention – CS+N) and 11 CS- presentations. All presentations related to Day 2 take place in context B. Day 3 is divided into two different components, all taking place in context A. First, each CS (CS+R, CS+N and CS-) is presented twice in an unreinforced manner in order to assess renewal. This is followed by the presentation of 3 shocks alone and 8 further unreinforced presentations of each CS (CS+R, CS+N and CS-) in order to evaluate reinstatement. Finally, on Day 30, all presentations take place in a new context, namely context C. In order to evaluate spontaneous recovery, 8 unreinforced presentations of each CS (CS+R, CS+N, CS-) are presented first. This is followed by 8 presentations of all CSs, with the two CS+s being reinforced 62.5% of the time. This second acquisition phase allows us to examine savings during re-acquisition.

Importantly, although the same spider is always used for the CS-, two different task versions were designed so that the two remaining spiders are alternated regarding whether they serve as CS+R or CS+N.

## 2. BODY

Subject recruitment: At of the end of the 02 year, we had recruited a total of 85 subjects. In the 03 year, we enrolled an additional 75 participants, for a total of 156 enrolled to date. These 75 additional participants have been selected from 159 screened by phone in the 03 year. Among them, 66 have completed all four testing sessions, and an additional 6 have yet to attend the follow-up visit. 13 completed the initial three visits but were subsequently lost to follow-up, 2 dropped out of their own accord before completing day 3, 8 were withdrawn from the study before completing day 3 due to various reasons (e.g., unreadable skin conductance levels, falling asleep, data collection error). The remaining 61 participants were screened out of the experiment due to failure to demonstrate differential conditioning in their first session. Importantly, in the 03 year we were significantly closer to our recruitment goal of two participants per week, as outlined in the Statement of Work, than in the 01 and 02 years.

Statement of Work:
***Experiment 1:*** **Reactivation plus propranolol (Completed).** This consisted of four experimental phases (laboratory visits) entailing three consecutive days and a fourth day one month later. Prior to beginning the experiment, normal human subjects who reported that they were afraid of spiders (but who did *not* meet any criteria for the spider phobia diagnosis) set their own level of electric stimulation (shock). They were then instructed to choose a level that was highly annoying but not painful. The experimenter started the stimulation at a very low, nearly imperceptible level and gradually increased the level until the subject said "stop." That level was then used throughout the experimental sessions.

Day 1. (Habituation, Acquisition). Two of three videos of moving tarantulas presented via high-definition large-screen television served as the two reinforced conditioned stimuli (CS+s; the to be CS+R and CS+N), and the third video as an unreinforced control stimulus (CS-.) Each CS+ presentation was sometimes followed by shock (i.e., reinforced); the CS- was never followed by shock. Day 1 consisted of two sequential components: a.) 2 unreinforced presentations of each CS (habituation), followed by b.) 8 presentations of each CS+, with 5 of

each CS+ presentations followed by shock (i.e., 63% reinforcement, acquisition) and 16 presentations of the CS-. All Day 1 CS presentations occurred within video Context A.

Day 2. (Intervention). The intervention consisted of 40 mg oral propranolol. This was followed 90 minutes later by a single, unreinforced presentation of one of the two CS+s, designated the reactivated CS+ (CS+R). The CS+ that was not presented on Day 2 is designated as the non-reactivated CS+ (CS+N). All Day 2 CS presentations took place within video Context B.

Day 3. (Renewal, Reinstatement) consisted of three sequential components: a.) 2 unreinforced presentations each of the CS+R, CS+N, and CS- (renewal test trials); b. 3 presentations of the US alone, and c.) 8 further unreinforced presentations each the CS+R, CS+N, and CS- (reinstatement test trials, also re-extinction). All Day 3 CS presentations occurred within video Context A.

Day 30. (Spontaneous Recovery/Renewal, Savings) consisted of two sequential components: a.) 8 unreinforced presentations each of the CS+R, CS+N, and CS- (spontaneous recovery test trials, also re-extinction); and b.) 8 presentations each of the two CS+s with 63% reinforcement, and the CS- (savings test trials during re-acquisition). All Day 30 CS presentations occurred within Context C.

Of the 156 participants who have been enrolled, 55 were assigned to this propranolol intervention.

**For full results and discussion of findings from Experiment 1, please see below.**

**Months 15-27** ***Experiment 2:*** **Reactivation with delayed extinction (Analyzing data).** This is the same as Experiment 1 except for Day 2. On Day 2, no drug is administered; instead, a single unreinforced presentation of the CS+R is followed 10 minutes later by a.) 10 further unreinforced CS+R presentations; b.) 11 unreinforced presentations of the CS+N; and c.) 11 CS- presentations. Of the 156 participants who have been enrolled, 88 were assigned to the delayed extinction intervention. We are in the process of analyzing data from the behavioral intervention, but do not yet have preliminary findings to report.

**Months 26-28.** ***Experiment 3:*** **Reactivation plus mifepristone (Underway).** This will be the same as Experiment 1 except for Day 2. On that day, 1800 mg oral mifepristone will be substituted for propranolol. Of the 156 participants who have been enrolled, 13 have been assigned to the current, mifepristone drug intervention. Data collection for the mifepristone intervention is ongoing, but numbers are too few for preliminary analyses. At present, there are no findings to discuss for this experiment.

**Months 29-31** ***Experiment 4*****: Reactivation plus a different pharmacological agent (Planned).** This will also be the same as Experiment 1 except for Day 2. On that day, a different agent will be substituted for propranolol. At this point the most likely choice is oxytocin, but the final selection will be dictated by the results obtained from the earlier experiments up to that point.

**Months 32-34: *Experiment 5:* Reactivation plus another pharmacological agent (Planned).** This will also be the same as Experiment 1 except for Day 2. On that day, another agent will be substituted for propranolol. At this point the most likely choice will be nabilone, but the final selection will be dictated by the results obtained from the earlier experiments up to that point.

**Months 35-36.** The final two months of the project will be devoted to data reduction and statistical analysis, and the preparation of publications.

## Experiment 1: Pre-reactivation propranolol fails to reduce skin conductance reactivity to fear-conditioned stimuli


### Abstract

Memory reconsolidation is the process by which a recalled memory must restabilize in order to persist. Pharmacologic blockade of this process has been demonstrated in fear-conditioned rodents and humans. Reconsolidation blockade may provide a means by which to reduce fearfulness in anxiety disorders such as post-traumatic stress disorder. Research examining the efficacy of potential interventions in clinical populations is challenging. Consequently, there exists the need for paradigms within which candidate reconsolidation blocking interventions can be readily tested. To this end, we used videos of tarantulas as biologically prepared stimuli to test the efficacy of pre-reactivation propranolol in blocking reconsolidation of conditioned fear in healthy individuals. Strong differential conditioning was observed during the acquisition phase, as measured by skin conductance reactivity. However, propranolol failed to reduce reactivity to the reactivated conditioned stimulus. These results are consistent with other recent findings, and point to a need for testing additional candidate drugs.


### Introduction

Once formed, a fear memory must stabilize in order to persist. This process, termed consolidation, occurs within a narrow window, i.e., minutes to hours, wherein the memory is labile and susceptible to intervention (Dudai, 2004; Walker, Brakefield, Hobson, & Stickgold, 2003). Potential clinical opportunities arise from this consolidation window, albeit narrow, including interventions for post-traumatic stress disorder (PTSD). Stress hormones may potentiate consolidation and thereby produce a memory trace that is easily activated and resistant to extinction (Pitman, 1989). Pharmacological agents, including beta-adrenergic antagonists, could limit the memory-modulating effects of these hormones (McGaugh, 2004) and in so doing attenuate consolidation, if administered during the consolidation window. This approach is complicated by the need to intervene before the memory has consolidated. Studies attempting to do this have produced mixed results (Hoge et al., 2012; Holmes, James, Coode-Bate, & Deeprose, 2009; Pitman et al., 2002; Vaiva et al., 2003).

As demonstrated by Nader and colleagues in animal research, reactivation (i.e., retrieval) of a consolidated memory returns it to a destabilized state, from which it has to be restabilized (*i.e.* reconsolidated) if it is to persist (Debiec & Ledoux, 2004; K Nader, Schafe, & Le Doux, 2000; Karim Nader & Einarsson, 2010). Interference with memory *reconsolidation* offers a more feasible clinical target, one that is governed by neurobiological processes similar to those of consolidation (Lee, Everitt, & Thomas, 2004), and is susceptible to pharmacological blockade at the level of stress hormone receptors (Debiec & Ledoux, 2004; Jin, Lu, Yang, Ma, & Li, 2007; Pitman et al.,

2011; Przybyslawski, Roullet, & Sara, 1999). Brunet and colleagues (Brunet et al., 2008) have extended these reconsolidation findings to individuals with PTSD. Within a double-blind randomized control trial, those participants who received propranolol prior to recall of their traumatic memory exhibited significantly lower overall physiological reactivity during a subsequent laboratory visit when they again recalled their traumatic experience.

Although clinical application remains the ultimate goal, there persists the need for a basic paradigm wherein candidate pharmacological agents can be more readily tested. Relatively few studies have investigated reconsolidation blockade in humans, and fewer still have done so in a normal (i.e., non-PTSD) population. Recent studies performed in healthy human subjects have helped to address this gap (Kindt, Soeter, & Vervliet, 2009; Soeter & Kindt, 2010, 2011). Soeter & Kindt (2010) used fear-relevant stimuli (i.e., pictures of spiders) in a differential fear conditioning paradigm with potentiated eye blink startle response serving as the CR. After first learning to associate one spider (CS+) with the shock, but not the other spider (CS-), participants received either propranolol or placebo in conjunction with a single reactivation trial of the CS+ alone, i.e., no shock. A third group of subjects received propranolol without memory reactivation. Finally, subjects viewed ten unreinforced presentations each of the CS+ and CS-. Participants in the placebo-reactivation and propranolol-non-reactivation groups showed strong eye blink responses to the CS+, compared to the CS-, suggesting preservation of the conditioned fear memory. In contrast, subjects in the propranolol-reactivation group showed comparably small eye blink responses to the CS+ and CS-, suggesting erasure of the fear memory. Furthermore, the eye blink response remained small, i.e., was not reinstated, for the propranolol-reactivation group in a subsequent reinstatement test. The investigators interpreted their findings as indicating blockade of reconsolidation of the conditioned fear response following memory reactivation by presentation of the CS+ accompanied with propranolol.

The findings of Soeter & Kindt (2010) suggest that: a) reconsolidation and its pharmacological blockade generalize to a non-clinical model of fear memory in humans, and b) propranolol is apparently efficacious in the abolishment of conditioned fear memories as measured by eye blink startle response. Unfortunately, the protocol also produces a floor effect, wherein there is a 100% reduction of the fear memory trace in the propranolol-reactivation condition. This floor effect precludes comparison of the relative strengths of candidate interventions; an intervention that is potentially more effective at reducing memory reconsolidation could not produce more than 100% reduction in this paradigm. In an attempt to overcome this limitation and build upon the collective findings of Kindt & Soeter, our aim was two-fold. We sought to create and validate an optimal Pavlovian fear conditioning paradigm that could be used to test the relative strengths of various drug and non-drug candidates for reconsolidation blockade. The paradigm must be more resistant to total blockade, and thereby exhibit significant but only partial (i.e., <100%) reduction of the fear memory. To this end, we employed more highly prepared CSs, more fear-sensitive subjects, and stronger CRs.

First, it has long been shown that certain classes of CSs, when paired with a US, produce a stronger fear CR, i.e., they are more "prepared" to enter into an association with the US (Mineka & Öhman, 2002). Kindt and colleagues (Kindt et al., 2009; Soeter & Kindt, 2010) used prepared CSs, viz., still pictures of spiders. We enhanced preparedness of the CSs by using 12-sec, high-definition video clips of three crawling tarantulas, each conspicuously different in appearance. Second, we limited enrollment to participants who scored in the upper half of the normal human distribution on the Spider Phobia Questionnaire-15 (Olatunji et al., 2009). However, we screened out anyone who endorsed symptoms of clinical spider phobia. Third, we required that participants show evidence of strong differential conditioning, as determined by a cutoff assigned

to CRs recorded during Day 1 acquisition. Participants with subthreshold CRs were withdrawn after Day 1. Soeter & Kindt (2010) also applied a less stringent cutoff (i.e., mean acquisition trials 7-8 CS+ > CS-).

We also aimed to test the efficacy of propranolol in blocking reconsolidation and reducing fear memory within this paradigm, as measured by changes in skin conductance (SC). Soeter & Kindt (2010) recorded both eye blink startle and SC responses and, while total abolishment of the differential eye blink response was observed, there was no reduction by propranolol of the conditioned SCR. Failure of the SC response to demonstrate reconsolidation blockade is a bit perplexing, given that this has been a widely used measure of human fear conditioning (see Boucsein, 2012). Moreover, it is not clear what impact the presentation of noise stimuli during the CS interval may have had on SCR, as these startle probes introduce an additional US, i.e. a 104 dB loud noise. In order to avoid the potential confound produced by the startle probes, we chose to only record SC responses.

## Methods

### Participants

Prior to enrollment, participants were screened by phone to verify a) absence of medical conditions that would contraindicate administration of propranolol, i.e., asthma, hypotension, diabetes, and b) presence of a manageable, non-phobic fear of spiders as determined by mid-range scores on the Spider Phobia Questionnaire 15 (SPQ-15; Olatunji et al., 2009) and phobia criteria extracted from the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-IV; First, Spitzer, Gibbon, & Williams, 1997). All participants underwent either a full SCID-IV or a set of screening criteria taken directly from the SCID-IV. Participants were free from current psychiatric disorders, serious medical or neurological conditions, brain injury, and current or past substance abuse. A urine drug screen verified the absence of illicit substances and psychotropic medications.

Fifty-five healthy participants (35F, 20M) were enrolled in the study. Of these, three were withdrawn before commencing the Day-1 procedure due to: unmeasurable (very low) SC levels (n=2), and presence of a current Axis I psychiatric disorder (n=1). An additional 28 were withdrawn after Day 1 due to: non-compliance (n = 1), data collection error (n = 1), drop out (n = 1), and failure to demonstrate adequate differential conditioning (n = 25; conditioning criteria are described below in Data Reduction). The remaining 24 (12F, 12M) who underwent study procedures on Days 1-3 had an average age of 22.6 years (SD = 3.2, range 18 to 31 years) and an average score on the SPQ-15 of 8.0 (SD = 1.8, range 6 to 11). Years of education ranged from 12 to 19 (M = 15.5, SD = 1.9); three participants failed to provide this information. Seven of the 24 participants were not included in Day 30 analyses due to: data collection error (n = 1), and being lost to follow-up (n = 6).

The study protocol was approved by the Partners Human Research Committee (PHRC), as well as the United States Army Medical Research and Material Command (USAMRMC) Human Research Protection Office (HRPO). All participants provided written informed consent prior to undergoing study procedures.

### Equipment and stimuli

Skin conductance analog signals were recorded using a Coulbourn Lab Linc V Series Human Measurement System (Coulbourn Instruments, Whitehall, PA) with a Coulbourn Isolated Skin Conductance Coupler (V71-23) through 8mm (sensor diameter) Ag/AgCl electrodes (In Vivo Metric; Healdsburg, CA) filled with an isotonic paste. Electrodes were separated by 14mm, as

determined by the width of the adhesive collar, and placed on the hypothenar surface of the subject's non-dominant hand in accordance with published guidelines (Boucsein et al., 2012; Fowles et al., 1981). The SC signal was sampled at 1000 hz and digitized by a Coulbourn Analog to Digital Converter (V19-16). A Cobalt notebook computer (IBM-compatible; Cobalt Computers, Whitehall, PA) with custom-designed software was used to record and store the digitized physiological signals.

Conditioned stimuli (CS) consisted of nine high-definition video clips (Virtually Better Inc., Decatur, GA) depicting one of three tarantulas occupying one of three contexts. Two of the three tarantulas always served as one of the two CS+s, either the to-be-reactivated CS+ (CS+R) or the to-be-non-reactivated CS+ (CS+N), and were paired with the unconditioned stimulus (US, shock) on day 1. The to-be CS+R served as the stimulus that was presented on day 2 after receiving the study medication; the to-be CS+N was not presented on day 2. The third tarantula always served as the CS-, which was not paired with the US and not presented on day 2. The three contexts within which the tarantulas appeared were a kitchen (A), bedroom (B) and office (C). The particular tarantula that served as either the CS+N or CS+R was counterbalanced across participants; the tarantula used to represent the CS- was the same across subjects.

The US was a 0.5-sec mild electric shock ranging in intensity (0.2 to 4.0 milliamperes) according to the level selected by the participant to be "highly annoying but not painful." The US was delivered using a Coulbourn Transcutaneous Aversive Finger Stimulator (E13-22) through shock electrodes attached to the middle segments of the 2$^{nd}$ and 3$^{rd}$ fingers on the hand opposite to that on which the SC recording electrodes were attached.

Video clips lasted 12 seconds: four seconds of context alone (i.e., no tarantula), followed by eight seconds of context plus tarantula. On reinforced trials, the US immediately followed the CS+. The intertrial interval consisted of a black screen and was randomized to last 16, 18, 20, 22, or 24 seconds. The procedure was implemented using E-Prime Professional 2.0 (Psychology Software Tools, Inc., Sharpsburg, PA).

### Procedure

As depicted in Figure 1, the procedure consisted of a differential fear-conditioning paradigm that entailed laboratory visits over three consecutive days and a follow-up visit at four weeks. On average, participants returned for the follow-up 30 days after their third visit (M = 29.9; SD = 2.7). On day 1, participants were instructed: *"Today, you will be viewing videos of spiders on the television, and you will receive electric shocks on your fingers after viewing some of the spiders. These shocks will be annoying, but not painful. We will also use electrodes on your palm to record how your body responds to this procedure."* Following these instruction, participants set the shock to a level they determined to be “highly annoying but not painful” (Orr et al., 2000). Participants were shown still images, i.e., screenshots, of the three tarantulas that would serve as CSs, accompanied by these instructions: *"During the experiment, it will be important that you are able to tell these spiders apart. To do this, try focusing on the legs. For this spider, note the alternating black and white stripe pattern. For this spider, note the orange highlights. For this spider, note that the legs are solid black."* Prior to beginning the procedure, the lights were dimmed and over-ear headphones placed on the participant to reduce ambient noise and enable communication with study staff in the next room. Participants were instructed to sit still in the chair, keep their eyes open, and be attentive to the stimuli presented on the screen. Next, there was a 5-min baseline period to record physiological levels.

Day 1 consisted of two sequential phases: 1) unreinforced presentations of each of the nine possible spider-context combinations in pseudorandom order (*habituation*), and 2) eight partially

reinforced, i.e., five of eight, presentations each of CS+R and CS+N, presented separately in blocks and interspersed pseudorandomly with eight presentations of CS- (*acquisition*). The order of presentation of CS+R and CS+N was counterbalanced across participants and all CS+R, CS+N, and CS- presentations during acquisition occurred within context A. Participants who did not meet the defined cutoff for demonstrating a differential conditioned response (see below) were withdrawn prior to day 2.

The procedures for days 2, 3, and the 4-week follow-up were largely the same as for day 1, with the following exceptions: a) the procedure for setting the level of shock was not repeated, as the shock level determined on the first visit was used for the remainder of the study, b) participants were only familiarized with images of the stimuli prior to undergoing the day 1 procedure, and c) rather than "will receive" as on day 1, participants were instructed that they "may or may not receive" electric shocks.

Day 2 consisted of the participant receiving a 40 mg oral dose of short-acting propranolol (Mylan Pharmaceuticals, Pittsburgh, PA) followed 90 minutes later by a single, unreinforced presentation of the CS+R (*reactivation*). Propranolol reaches peak plasma levels approximately 90 minutes after ingestion (Gilman & Goodman, 1996). Reactivation of the CS+R occurred in context B. On day 2, blood pressure was manually auscultated immediately prior to administration of propranolol, as well as immediately following the single presentation of CS+R. Paired t-tests were used to compare the time points and thus verify the expected physiological effects of propranolol. A significant reduction in systolic ($t(19) = 5.44$, $p < .0001$), but not diastolic ($t(19) = .68$, $p = .25$) blood pressure was observed. Also, there was a significant decrease in pulse measured at those same time points ($t(19) = 7.04$, $p < .0001$).

Day 3 consisted of three phases: 1) two unreinforced presentations each of the CS+R, CS+N, and CS- pseudorandomly interspersed (*renewal test*), and 2) three unsignalled presentations of the US alone, followed by 3) ten unreinforced presentations each of the CS+R, CS+N, and CS- pseudorandomly interespersed (*reinstatement test trials* and *extinction*). All presentations of the CSs occurred in context A. As on day 1, ordering of CS+R and CS+N presentations was counterbalanced across subjects.

The four-week follow-up consisted of two phases: 1) ten unreinforced presentations each of the CS+R, CS+N, and CS- pseudorandomly interspersed (*spontaneous recovery test* and *re-extinction*), followed by 2) eight presentations each of CS+R and CS+N presented in successive blocks and interspersed with eight CS- trials for the respective blocks as was done on day 1 (*re-acquisition/savings test*). All stimuli were presented in context C during this visit and the CS+R block of trials was presented first.

**Physiological Measures and Data Reduction**

As previously described (Milad, Orr, Pitman, & Rauch, 2005; Orr et al., 2000), a SC response for the CS interval was calculated for each trial by subtracting the mean SC level during the two sec before CS onset (context alone presentation) from the peak SC level during the eight sec CS duration. These SCR values thus reflect change in skin conductance beyond that resulting from presentation of context alone. A square root transformation was applied to the absolute value of each SCR, followed by replacement of the +/- sign, prior to statistical analysis.

For day 1, the untransformed SCR data were examined to determine whether a definable differential SCR was observed for *both* the CS+R and CS+N during the acquisition phase. Averaging SCRs across respective CS+R, CS+N, and CS- trials, we calculated a difference score between the CS+R and its respective CS- trials and between the CS+N and its respective CS-

trials. A cutoff of .1μS was applied to each difference score. Participants with one or both difference scores below this cutoff were withdrawn from the study prior to Day 2.

## Results

Mixed-model, repeated measures analysis of variance (ANOVA) served as the primary statistical model, with Participants as a random effect, Stimulus (CS+R, CS+N, CS-) as a within-participants effect, and Trials as the repeated measure.

### Acquisition Phase (day 1).

As seen in Table 1 and Figure 2, panel A, during the acquisition phase, the CS+R and CS+N demonstrated comparably larger SCRs, compared to their respective CS- trials. The magnitude of the SCRs did not differ between CS+R and CS+N trials. However, there was a significant stimulus by trial interaction ($F(7,161) = 2.19$, $p = .038$) when comparing CS+R to CS-.

SC responses for the US interval, which represented the unconditioned response (UR), were calculated and plotted for the acquisition phase (see Figure 3). Because SCR onset has a known latency of 1-2 s (Edelberg, 1967), the 1-s interval immediately following US onset was used as the baseline for calculating the SC UR. Mean SC level during the 1-s interval immediately following US onset was subtracted from the peak SC level within the 6-s interval following US onset. A square root transformation was applied to the URs, as was done with CRs. Both CS+R and CS+N trials produced larger SCRs during the US interval, compared to CS- trials ($F(1,23) = 275.0$, $p < .0001$; $F(1,23) = 237.3$, $p < .0001$, respectively). There was also a significant Stimulus x Trial interactions ($F(7,161) = 27.5$, $p < .0001$; $F(7,161) = 31.3$, $p < .0001$, respectively).

### Renewal Phase (day 3).

As can be seen in Table 1 and Figure 2, panel B, SCRs to the CS+R and CS+N were significantly larger than to the CS-, demonstrating a persistence of the conditioned fear response. Contrary to our hypothesis, there was no significant difference in the magnitude of the SCR to the CS+R versus CS+N. If anything, the magnitudes of SCRs to the CS+R were slightly larger than the SCRs to CS+N (see Figure 2, panel B).

### Reinstatement Phase (day 3).

Responses to the CSs during the reinstatement phase, which immediately followed the unsignalled shock presentations, were initially examined across the ten presentations of each CS using a mixed-model repeated measures ANOVA with two factors: Stimulus (CS+R, CS+N, CS-) and Trials. There was a non-significant trend for an effect of Stimulus ($F(2, 46) = 2.84$, $p = .069$), as well as a significant Stimulus x Trials interaction ($F(18, 414) = 1.72$, $p = .034$). In order to decompose the interaction effect, we performed pair-wise comparisons of the CS+R, CS+N, and CS- trials. As can be seen in Table 1, there was a near significant main effect for the comparison of SCR magnitude for the CS+R and CS- trials, but not for the comparisons between CS+N and CS- trials and between CS+R and CS+N trials. Significant Stimulus x Trial interactions were observed for comparisons between the CS+N and CS- ($F(9, 207) = 1.99$, $p = .042$) and between the CS+R and CS+N ($F(9, 207) = 1.97$, $p = .045$), but not between the CS+R and CS- ($F(9, 207) = 1.18$, $p = .309$). As can be seen in Figure 2, the significant interaction for the comparison between CS+R and CS+ N trials reflected somewhat larger SCRs to initial CS+R presentations and then a reversal whereby there were somewhat larger SCRs to the later CS+N presentations. We also performed an analysis that only considered the first two presentations of each CS during

the reinstatement phase, so as to avoid the potential dampening of differences in reactivity to the CSs due to rapid extinction. The SCRs to CS+R and CS+N presentations were significantly larger than SCRs to CS- presentations (respectively: $F(1, 23) = 7.78$, $p = .010$); $F(1, 23) = 7.48$, $p = .012$); however, there was no significant difference between SCRs to the CS+R and CS+N presentations ($F(1, 23) < 1$, $p = .760$).

**Re-extinction phase (4 weeks).**

Responses to the CS+R, CS+N and CS- trials during the re-extinction phase were analyzed using mixed-model repeated measures ANOVA with two factors: Stimulus (CS+R, CS+N, CS-) and Trials. There was no significant Stimulus main effect ($F(2, 32) = 1.21$, $p = .312$), as well as no significant Stimulus x Trials interaction ($F(18, 288) = 1.45$, $p = .106$) (see Figure 2, Panel C).

**Re-acquisition phase (4 weeks).**

For re-acquisition, as seen in Table 1 and Figure 2, Panel C, we observed a pattern similar to that of acquisition on day 1. Reactivity to CS+R and CS+N was markedly stronger when compared to their respective CS- trials, but there was no significant difference in the magnitudes of SCRs to the CS+R and CS+N. There was a significant Stimulus x Trial interaction when comparing CS+R to CS- ($F(7, 111) = 5.00$, $p < .0001$) and CS+R to CS+N ($F(7, 111) = 2.53$, $p = .019$).

## Discussion

Brief clips of moving tarantulas depicted within various contexts served as the conditioned stimuli for fear-conditioned SCRs. The differential conditioning procedure produced robust SCRs to the stimuli that would subsequently serve as the reactivated (CS+R) and non-reactivated (CS+N) cues, by which reconsolidation blockade of conditioned fear responses was assessed. Differential SCRs to the CS+R and CS+N, compared to their respective CS- presentations, remained largely intact and significant across follow-up testing of renewal and reinstatement (day 3) and reacquisition (4-weeks).

Efficacy of pre-reactivation propranolol on reconsolidation blockade was tested on the day following fear conditioning by means of a single presentation of one of the two CS+s (CS+R) after the participant had received a single dose of propranolol. We found that propranolol had no measurable effect on reconsolidation blockade of the fear-conditioned SCR. No significant differences between SCRs to the reactivated and non-reactivated CS+ were observed on either of the post-reactivation visits. If anything, the pattern of SCRs was opposite to our hypothesis; i.e., the CS+R tended to elicit stronger SCRs than did CS+N, particularly on day 3. Results indicate that administration of propranolol prior to reactivation of the CS+R failed to selectively block reconsolidation of the targeted fear memory trace.

Our findings are consistent with those reported by Soeter & Kindt (2010) for SCR, wherein they employed fear-relevant stimuli within a differential fear-conditioning paradigm to assess the impact of pre-reactivation propranolol on SCR, as well as fear-potentiated startle. Soeter and Kindt observed a selective and complete abolishment of the fear-potentiated startle response, but no significant effect on SCR. Studies that have examined the effects of benzodiazepines on acoustic startle responses have also observed reductions in fear-potentiated startle in the absence of an effect on SCRs (e.g., Graham et al., 2005). The negative findings for SCR, yet positive findings for fear-potentiated startle, are noteworthy. Soeter and Kindt interpreted this discrepancy as indicative of distinct neural substrates that govern separate memory systems, i.e., declarative and procedural. Specifically, they suggested that SCR reflects declarative memory governed by the hippocampal complex, whereas fear-potentiated startle

measures fear that is principally linked to amygdala activity. The finding that subjective reports of US expectancy (i.e., declarative knowledge) were also unaffected by propranolol was taken by Soeter and Kindt as further support that the fear-conditioned SCR reflected declarative memory.

Contrary to Soeter and Kindt's (2010) interpretation, there is substantial evidence that fear-conditioned SCRs represent something more than declarative knowledge. For example, multiple studies have clearly demonstrated fear-conditioned SCRs to masked stimuli (see Esteves, Parra, Dimberg, & Ohman, 1994; Flykt, Esteves, & Ohman, 2007), suggesting that SCRs can also be linked to non-declarative memory systems. Furthermore, conditioned SCRs have been linked to metabolic activity in the fear circuit. In a study of human fear conditioning, Agren and colleagues (Agren et al., 2012) manipulated extinction by altering the amount of time following the first extinction trial, so as to examine the effect of a short versus long delay on reconsolidation blockade of the fear memory. Participants underwent reactivation of the CR by a single presentation of the CS that lasted two minutes, followed by extinction trials that were delayed either ten minutes (active) or six hours (control). Magnitudes of SCRs were significantly reduced in the active, compared to control, group and this reduction coincided with attenuation of blood oxygen level dependent (BOLD) activity in the basolateral amygdala. Furthermore, SCRs and BOLD activity of the amygdala were positively correlated in the days following delayed extinction for both groups.

In another study that examined SCR during neuroimaging, Williams et al. (2001) simultaneously recorded SC and fMRI while participants viewed stimuli consisting of neutral control and fearful faces. Consistent with Agren et al. (2012), elicitation of an SCR by the fear stimuli coincided with heightened amygdala activity. Furthermore, Williams et al. only observed increased hippocampal activity when the fear stimuli *did not* elicit an SCR. Taken together, the above findings suggest that SCRs: a) are associated with activity of the amygdala complex, b) are not necessarily associated with hippocampal activity when triggered by fear-relevant stimuli, and c) can reflect processes in the absence of declarative knowledge.

Why then does fear-potentiated startle, but not SC, respond to reconsolidation blockade by propranolol in humans? Skin conductance is considered to be a relatively pure measure of sympathetic activity (Wallin, 1981) and those stimuli that activate the sympathetic nervous system, i.e., increase sympathetic arousal, will produce an SCR. The acoustic startle reflex has been shown to be influenced by emotional valence, such that it is augmented and diminished in the presence of emotionally negative and positive stimuli, respectively (e.g., Hamm, Greenwald, Bradley, Cuthbert, & Lang, 1991). In an early study of startle potentiation in humans, Bradley, Cuthbert and Lang (Bradley, Cuthbert, & Lang, 1990) observed that, whereas the acoustic startle response was determined by stimulus valence, SCR reflected the amount of arousal generated by the stimulus. More specifically, the startle response was augmented in the presence of an emotionally negative stimulus and diminished in the presence of a positive stimulus, while SCR was increased to both positive and negative stimuli. The different findings for fear-potentiated startle and SCR pertaining to reconsolidation blockade suggest that propranolol can interfere with reconsolidation of the negative emotional valence associated with a fear-conditioned memory, but does not block reconsolidation of the sympathetic arousal generated by the stimulus. Thus, the process that maintains the arousal component of a conditioned fear response appears to be resistant to reconsolidation blockade by propranolol in healthy individuals.

Clinical studies that have examined the ability of propranolol to interfere with reconsolidation of a traumatic memory in individuals diagnosed with PTSD have provided some support for its efficacy (Brunet et al., 2014, 2008). For example, Brunet and colleagues (Brunet et al., 2008) found that propranolol administered shortly after reactivation of an individual's

traumatic memory reduced psychophysiologic responding, which included a measure of SC reactivity, during subsequent script-driven traumatic imagery in individuals with PTSD, compared to a placebo control group. It is worth noting that this clinical work used a more prolonged re-activation of the fear cue, as well as a combination of short-acting and long-acting propranolol, compared to the present study and those of Soeter and Kindt (Soeter & Kindt, 2010, 2011), which used a relatively brief presentation of the fear CS and a single 40 mg dose of short-acting propranolol. It may be that a longer CS presentation and/or more enduring propranolol dosage may be needed to interfere with reconsolidation of the fear-conditioned SCR. If propranolol is to be pursued as a candidate intervention in reconsolidation blockade, future conditioning studies might consider increasing the dosage across all participants (i.e., >40mg) or adjusting the dosage based on participant weight, so as to amplify the antagonistic effects of the drug.

It is possible that in our efforts to generate stronger differential conditioning that would be more resistant to a floor effect (i.e., use of an SPQ-15 cutoff score, more salient stimuli, and a stringent conditioning cutoff score) we actually produced conditioning that was highly resistant to noradrenergic blockade. It may also be that the day 2 reactivation trial actually potentiated SCRs to CS+R and this potentiation masked the effects of noradrenergic blockade. Although this seems unlikely, a placebo condition would have helped determine whether such potentiation had occurred.

A primary goal of the present work was to develop a strong conditioning model that could be used to test the potential efficacy of new candidate reconsolidation-blockade interventions in readily available normal samples before conducting more expensive and challenging clinical trials. It seems desirable to develop a model that will produce the most resilient fear conditioning that is ethically justifiable, particularly if the results are to be translated to a disorder characterized by over-consolidated fear memories, as is PTSD (Pitman, 1989). We believe that our novel paradigm fulfills these criteria and represents an excellent model within which to test and compare reconsolidation blocking interventions. However, future investigations must also consider the observed discrepancies in how different indices of fear conditioning, such as fear-potentiated startle and SCR, respond to reconsolidation blockade. Moreover, we should strive to reconcile contradicting results across comparable tests of reconsolidation blockade (e.g., Kindt & Soeter, 2013 and Schiller et al., 2010). Efforts must be directed to better understand the cause and clinical impact of such findings.

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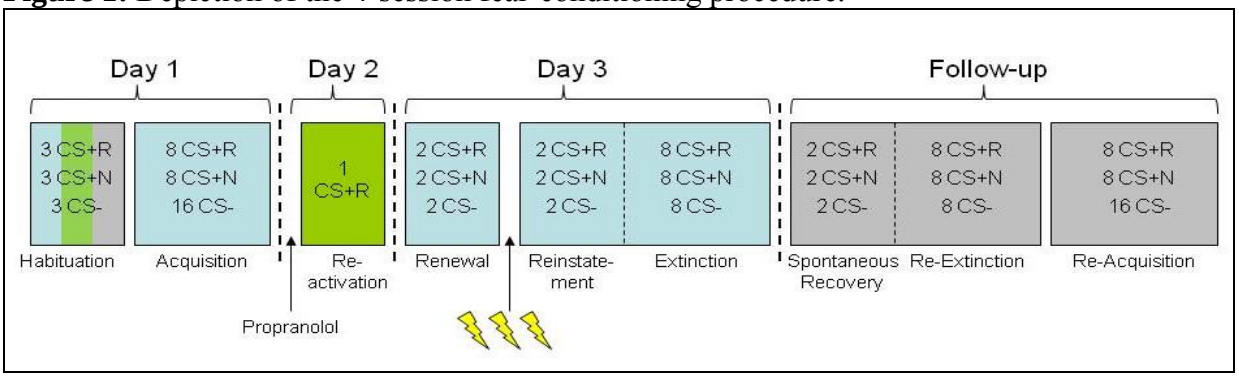
## Tables

**Table 1:** Results of mixed-model repeated measures ANOVA of SCR (√μS) for the CS interval.

| Day | Test Phase | Comparison | DF | F-value | p |
|---|---|---|---|---|---|
| Day 1 | Acquisition | CS+R vs CS- | 1, 23 | 87.4 | <.0001 |
| | | CS+N vs CS- | | 43.71 | <.0001 |
| | | CS+R vs CS+N | | 1.11 | 0.3029 |
| Day 3 | Renewal | CS+R vs CS- | 1, 23 | 21.09 | 0.0001 |
| | | CS+N vs CS- | | 14.85 | 0.0008 |
| | | CS+R vs CS+N | | <1 | NS |
| | Reinstatement (2 trials) | CS+R vs CS- | 1, 23 | 7.78 | 0.0104 |
| | | CS+N vs CS- | | 7.48 | 0.0118 |
| | | CS+R vs CS+N | | <1 | NS |
| | Reinstatement (10 trials) | CS+R vs CS- | 1, 23 | 3.73 | 0.0659 |
| | | CS+N vs CS- | | 2.9 | 0.1022 |
| | | CS+R vs CS+N | | <1 | NS |
| 4 Weeks | Re-acquisition | CS+R vs CS- | 1,17 | 19.74 | 0.0004 |
| | | CS+N vs CS- | | 9.42 | 0.0073 |
| | | CS+R vs CS+N | | <1 | NS |

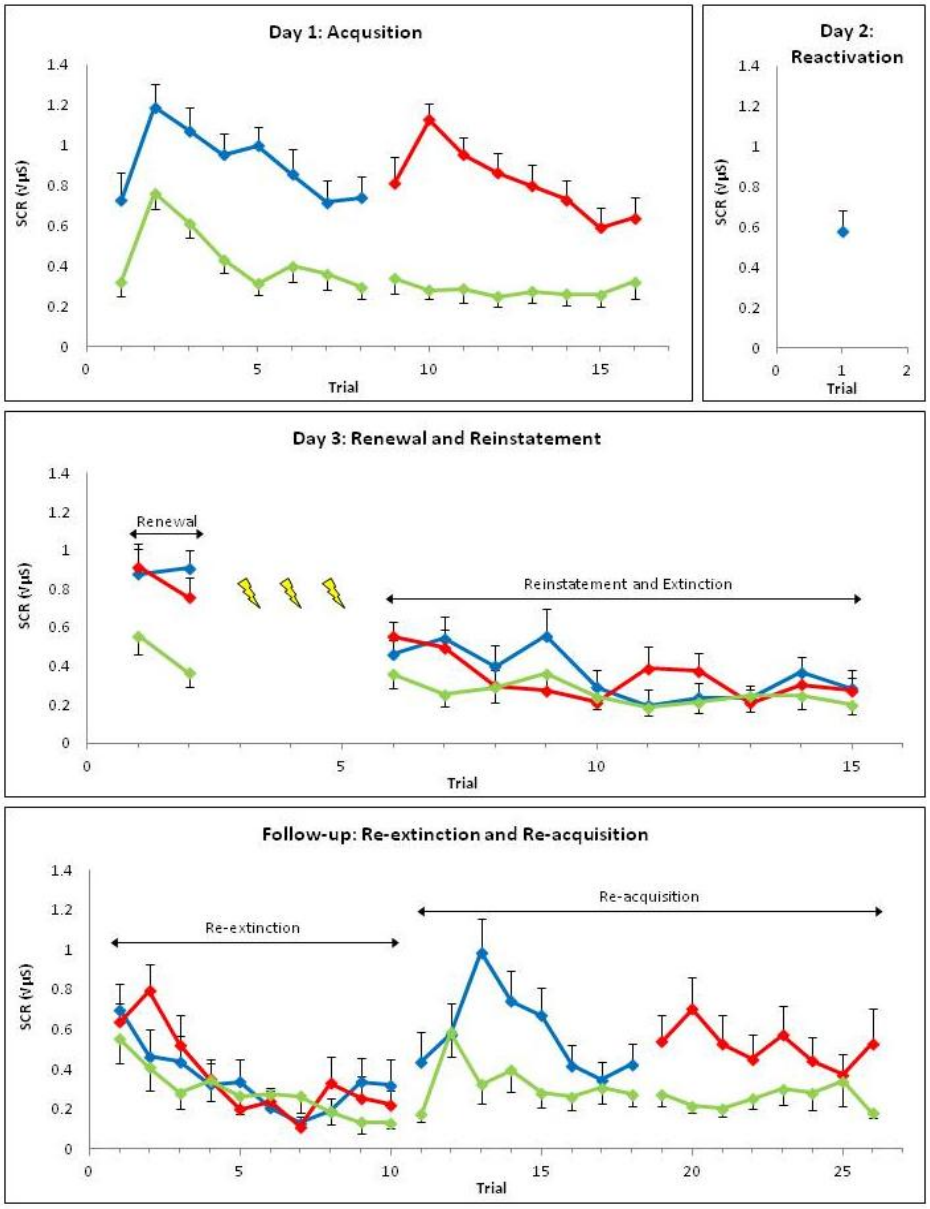
## Figures

**Figure 1:** Depiction of the 4-session fear-conditioning procedure.



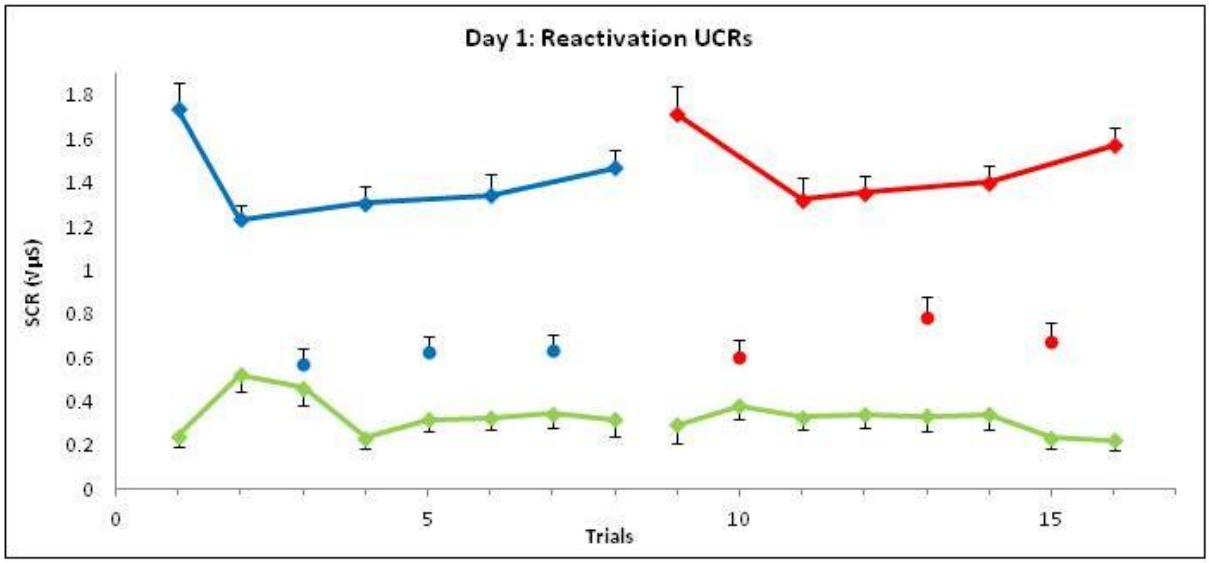

**Note**: Fear conditioning occurred on day 1. A single dose of 40 mg of propranolol was administered on day 2, followed by reactivation of one of the two the CS+s (CS+R). On day 3 and the 4-week follow-up visit, post-intervention reactivity to the conditioned stimuli was tested. CS+R = CS+ to-be-reactivated; CS+N = CS+ non-reactivated.. Lightning bolts represent unsignalled presentations of the US alone. Shading colors represent the context in which the stimuli were presented: blue = A, green = B, grey = C. CS+R and CS+N acquisition and re-acquisition trials occurred in blocks as described in the text.

**Figure 2.** Group mean skin conductance responses to CS+R, CS+N, and CS- trials for the Acquisition (day 1), Reactivation (day 2), Renewal and Reinstatement (day 3), and Re-extinction and Re-acquisition (4 weeks) phases.




**Note:** Bars represent standard errors. Color represents stimulus: blue = CS+R, red = CS+N, green = CS-. Lightning bolts represent unsignalled presentations of the US alone. SCR = skin conductance response.

**Figure 3.** Group mean skin conductance responses during US interval following CS+R, CS+N, and CS- trials for the Acquisition phase (day 1).




**Note:** Bars represent standard errors. Color represents stimulus: blue = CS+R, red = CS+N, green = CS-. Unconnected circles represent unreinforced (i.e., no shock) CS+ trials. SCR = skin conductance response.

## 3. KEY RESEARCH ACCOMPLISHMENTS

Based on our statement of work, we have met the proposed objectives. The following are the main steps that have been accomplished over the last year:

-Finalized analyses from Experiment 1, propranolol intervention.

-Completed full manuscript for propranolol intervention (see above), with anticipated submission to *Psychophysiology* this month (i.e., Feb '14).

-Presented poster at Society for Biological Psychiatry conference in May '13. Poster nominated for "Top Poster Award," with only 10% of posters nominated (see Appendix).

-Completed recruitment for Experiment 2, behavioral (i.e., delayed extinction) intervention, with n=24 exhibiting sufficient SCRs to be included in data analyses.

-Initial recruitment of 13 participants for Experiment 3, mifepristone intervention.

-Requested and received a 1-year no cost grant extension

## 4. REPORTABLE OUTCOMES

As mentioned under accomplishments, preliminary findings were initially presented as a poster at SOBP ’13 (May ’13). A manuscript draft has also been completed and we anticipate submission to *Psychophysiology* later this month.

## 5. CONCLUSION

The data and findings obtained to date suggest that our goal of creating a modified fear-conditioning paradigm that is free from intervention floor effects associated with blockade of memory reconsolidation has been achieved. In contrast to the success of the paradigm itself, our finalized results convincingly suggest that 40mg of propranolol does not block reconsolidation of a conditioned fear memory in healthy individuals. Our focus is now shifted to data analysis for the behavioral-intervention study, preparation of a manuscript reflecting these findings, and testing of pre-reactivation mifepristone in blocking fear-memory reconsolidation.

## 6. REFERENCES

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# 7. APPENDIX

# Pre-Reactivation Propranolol Fails to Reduce Skin Conductance Reactivity to Fear-Conditioned Stimuli

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## Introduction

Reactivation of a consolidated memory may return it to a labile state from which it must be restabilized in order to persist. Blocking this reconsolidation process offers novel avenues for treatment of post-traumatic stress disorder. However, recent efforts in normal humans completely abolished fear responses, thus precluding a comparison of the relative efficacies of the interventions. *We aimed to:*

1. Establish an experimental assay in the form of an optimal Pavlovian differential fear conditioning paradigm, within which to compare the relative strengths of behavioral and pharmacological reconsolidation-based interventions.
2. Determine whether the β-blocker propranolol, administered pre-reactivation, blocks reconsolidation of fear memory as evidenced by reduced sympathetic reactivity.

## Methods

• Differential conditioning paradigm utilizes: a) biologically-prepared video stimuli (i.e. tarantulas), and b) sensitive subject population (i.e. healthy, 18-35, afraid of spiders).

• 4 study visits

• *Day 1* (Context A): Conditioning with three tarantula stimuli: CS+, to-be Reactivated (CS+R) and CS+, not to be Reactivated (CS+N) are both paired with shock; CS- is not.


*Figure 1:* Still images of high-definition video tarantula stimuli. The two labeled "CS+" act as either CS+R or CS+N (randomized by participant). The third- is always the CS-. All images taken from a single context.

• *Day 2* (Context B): 40mg propranolol followed 90 minutes later by a single unreinforced reactivation of CS+R.

• *Day 3* (Context A): Tests for renewal and reinstatement, which probe the latent fear memory.

• *Day 30* (Context C): Tests for spontaneous recovery and savings (re-acquisition), which probe the latent fear memory.

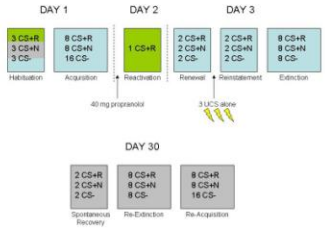



*Figure 2:* Protocol consists of four visits over one month. Boxes indicate the phase of the conditioning paradigm. Colors represent the three contexts (i.e. A, B, and C) in which stimuli are presented.

## Results

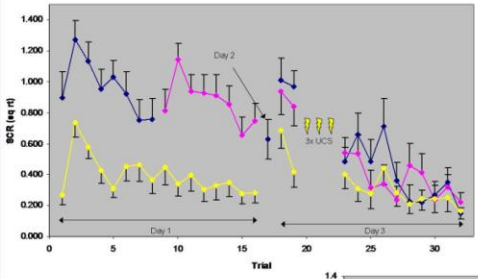
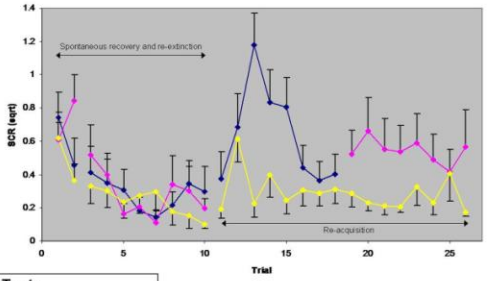



*Figure 3:* Days 1-3. Mean skin conductance response (μS) by trial across subjects. Shown are participants screened for strong differential conditioning on day 1 (n=18). Blue=CS+R, Pink=CS+N, Yellow=CS-. Standard error bars are included.

*Figure 4:* Day 30. Mean skin conductance response (μS) by trial across subjects. Shown are participants screened for strong differential conditioning on day 1 (n=14). Blue = CS+R, Pink = CS+N, Yellow = CS-. Standard error bars are included.




| | Statistical Tests | |
|---|---|---|
| Day 1 | **Acquisition (n=19; 8 trials)** | |
| | CS+R v CS- : F(1,18) = 66.09** | |
| | CS+N v CS- : F(1,18) = 38.44** | |
| | CS+R v CS+N : F(1,18) < 1 | |
| Day 3 | **Renewal (n=19; 2 trials)** | **Reinstatement (n=19; 2 trials)** |
| | CS+R v CS- : F(1,18) = 14.79** | CS+R v CS- : F(1,18) = 6.39* |
| | CS+N v CS- : F(1,18) = 10.26** | CS+N v CS- : F(1,18) = 4.58* |
| | CS+R v CS+N : F(1,18) < 1 | CS+R v CS+N : F(1,18) < 1 |
| Day 30 | **Spont. Recov. (n=15; 2 trials)** | **Savings (n=14; 8 trials)** |
| | CS+R v CS- : F(1,14) < 1 | CS+R v CS- : F(1,13) = 8.69* |
| | CS+N v CS- : F(1,14) = 6.66* | CS+N v CS- : F(1,13) = 5.21* |
| | CS+R v CS+N : F(1,14) < 1 | CS+R v CS+N : F(1,13) < 1 |

*Table 1:* ANOVA main effect of stimulus arranged by test and comparison. Acquisition takes place on day 1, whereas renewal/ reinstatement take place on day 3, and spontaneous recovery/ re-acquisition take place on day 30. Asterisks indicate significance: *<.05, **<.01).

## Discussion

• Novel fear-conditioning paradigm elicits strong differential conditioning utilizing biologically prepared stimuli and sensitive subject population.

• Probes for latent fear memory (i.e. renewal, reinstatement, spontaneous recovery, and savings) showed comparable reactivity to CS+R and CS+N.

• 40mg pre-reactivation propranolol failed to significantly reduce conditioned fear *as measured by skin conductance reactivity* to CS+R compared to CS+N. In terms of propranolol, we were unsuccessful in developing a useful experimental assay

• Replicates previous negative results obtained using skin conductance (SC). Fear-potentiated startle (FPS) has yielded positive results, but it wasn't studied here. However, FPS may be less relevant than SC to posttraumatic stress disorder.

## References

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